Sulfhydryl Oxidation Reduces Hippocampal Susceptibility to Hypoxia-Induced Spreading Depression by Activating BK Channels

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Hepp, Sebastian, Florian J. Gerich, and Michael Müller. Sulfhydryl oxidation reduces hippocampal susceptibility to hypoxia-induced spreading depression by activating BK channels. J Neurophysiol 94: 1091–1103, 2005; doi:10.1152/jn.00291.2005. The cytosolic redox status modulates ion channels and receptors by oxidizing/reducing their sulfhydryl (SH) groups. We therefore analyzed to what degree SH modulation affects hippocampal susceptibility to hypoxia. In rat hippocampal slices, severe hypoxia caused a massive depolarization of CA1 neurons and a negative shift of the extracellular DC potential, the characteristic sign of hypoxia-induced spreading depression (HSD). Oxidizing SH groups by 5,5'-dithiobis 2-nitrobenzoic acid (DTNB, 2 mM) postponed HSD by 30%, whereas their reduction by 1,4-dithio-dl-threitol (DTT, 2 mM) or alkylation by N-ethylmaleimide (500 μM) hastened HSD onset. The DTNB-induced postponement of HSD was not affected by tolbutamide (200 μM), dl-2-amino-5-phosphonovaleric acid (150 μM), or 6-cyano-7-nitroquinolxalin 2,3-dione (25 μM). It was abolished, however, by Ni²⁺ (2 mM), withdrawal of extracellular Ca²⁺, charybdotoxin (25 nM), and tiberiotoxin (50 nM). In CA1 neurons DTNB induced a moderate hyperpolarization, blocked spontaneous spike discharges and postponed the massive hypoxic depolarization. DTT induced burst firing, depolarized glial cells, and hastened the onset of the massive hypoxic depolarization. Schaffer-collateral/CA1 synapses were blocked by DTT but not by DTNB; axonal conduction remained intact. Mitochondria did not markedly respond to DTNB or DTT. While the targets of DTT are less clear, the postponement of HSD by DTNB indicates that sulfhydryl oxidation increases the tolerance of hippocampal tissue slices against hypoxia. We identified as the underlying mechanism the activation of BK channels in a Ca²⁺-sensitive manner. Accordingly, ionic disregulation and the loss of membrane potential occur later or might even be prevented during short-term insults. Therefore well-directed oxidation of SH groups could mediate neuroprotection.

INTRODUCTION

Reactive oxygen species (ROS) are mostly produced by the mitochondrial respiratory chain (Boveris and Chance 1973). Accordingly, changes in mitochondrial activity affect the cytosolic redox status, which is determined by the cellular H₂O₂ content as well as the two redox couples NAD⁺/NADH and reduced/oxidized glutathione (Dröge 2001). It is now clear that ROS are not only involved in cellular damage resulting from metabolic disturbances (Chan 2001), status epilepticus (Koç et al. 2004, 2005), persistent Na⁺ channels (Hammarström and Gage 2000), K⁺ channels (Seutin et al. 1995), and also Schaffer collateral/CA1 neuron synapses are modulated by H₂O₂ (Pellem 1987). Accordingly, there are various possibilities for redox modulation to control neuronal excitability. However, so far it is unknown to what degree redox signaling is involved in the response of neurons and glia to hypoxic insults such as anoxia and ischemia. Could cellular redox signaling possibly be part of the oxygen-sensing process in hippocampal neurons and trigger their immediate response to oxygen withdrawal? Could redox signaling even modulate the susceptibility of neural tissue against metabolic insults, could it possibly bear neuroprotective potential?

To address these questions, we focused on the very end of the redox signaling cascade, the SH groups: we analyzed to what degree the susceptibility of the hippocampal CA1 field toward anoxic insults, e.g., the generation of hypoxia-induced spreading depression (HSD), is affected by the modulation of SH groups. The specific targeting of SH groups was achieved by the SH oxidizing agent 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), the SH-reducing drug 1,4-dithio-dl-threitol (DTT), and the SH-alkylating compound N-ethylmaleimide (NEM) (for review, see Lipson et al. 2002). The effects of these compounds were analyzed on the network level, the cellular level, and on the subcellular level.

Some of the results have been published as abstracts (Hepp et al. 2004, 2005).

METHODS

Preparation

Hippocampal tissue slices were prepared from ether-anesthetized Sprague-Dawley rats of 150–300 g body wt (4–8 wk old, mostly males). After decapitation, the brain was rapidly removed from the skull and placed in chilled artificial cerebrospinal fluid (ACSF) for 1–2 min. The two hemispheres were separated, and 400-μm slices...
were cut using a tissue slicer (Campden Instruments, 752M Vibrosection). On a few occasions, the hippocampus was first isolated and then chopped (400 µm slices) using a custom-made tissue chopper. Slices were transferred to an interface recording chamber of the Oslo style and were left undisturbed for ≥90 min. The recording chamber was kept at a temperature of 35–36°C. It was continuously aerated with 95% O₂-5% CO₂ (400 ml/min), and perfused with oxygenated ACSF (3–4 ml/min). Hypoxia was induced by switching the chamber’s gas supply to 95% N₂-5% CO₂. To protect the slices from drying out and to prevent oxygenation from the air during hypoxic episodes, the slice chamber was covered by a lid with a small (2 cm²) opening for the positioning of the electrodes.

Cell cultures of hippocampal neurons were prepared from 2- to 4-day-old Sprague-Dawley rats. After decapitation, the brain was removed and placed in ice-cold HBSS (Hanks’ balanced-salt solution) containing 20% FCS (Biochrom). The dentate gyrus region was removed from the isolated hippocampi, and the remaining tissue was cut into smaller pieces and trypsinized (5 mg/ml) for 10 min at 37°C. Cells were then dissociated by gentle trituration, and the suspension was centrifuged for 10 min (1,500 rpm, 4°C). The pellet was re-suspended and plated on Matrigel (BD Biosciences)-coated glass coverslips, which were transferred to four-well culture plates (Nunc). Cultures were incubated at 37°C in a humidified, 5% CO₂-containing atmosphere. After 24 h, half of the 600 µl medium in each well was exchanged with growth-medium containing 4 µM cytosine arabinoside (Sigma-Aldrich). Cultures were kept for 2–2 wk, refreshing medium and growth factors after 4 days. Within 2–3 days in culture, cells fully recovered well-pronounced dendritic processes and were suitable for experiments; most experiments were performed between 3 and 7 days in culture.

Solutions

Chemicals, unless otherwise mentioned, were obtained from Sigma-Aldrich. The ACSF had the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 dextrose; aerated with 95% O₂-5% CO₂ to adjust pH to 7.4.

Minimum essential cell culture medium (MEM medium, Invitrogen) was supplemented with 5 mg/ml glucose, 0.2 mg/ml NaHCO₃, and 0.1 mg/ml transferrin (Calbiochem). For initial plating, it also contained 10% FCS (fetal calf serum), 2 mM L-glutamine, and 25 µg/ml insulin. The medium used after day 4 in culture contained 5% FCS, 0.5 mM L-glutamine, 20 µg/ml B27 50X supplement (Invitrogen), and 100 µg/ml penicillin-streptomycin (Biochrom). DTNB, DTT, and tetraethylammonium chloride (TEA) were directly dissolved in ACSF. DL-2-amino-5-phosphonovaleric acid (DL-AP5, Tocris) was prepared as 100 mM stock solution in 1 M NaOH and was kept frozen. Rhodamine 123 (Molecular Probes) was dissolved in absolute EtOH (20 mg/ml). Charybdotoxin (Sigma-Aldrich and Alomone Labs) and iberiotoxin (Alomone Labs) were dissolved in 100 mM NaCl, 10 mM HEPES plus 0.1% BSA. Paxilline, 6-cyano-7-nitroquinoxaline-2,3-dione (CQX), and tobutamide were dissolved in DMSO as 10, 50, and 100 mM stocks, respectively, and stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO. Solutions were stored at 4°C. NEM was prepared freshly as 250 mM stock in DMSO.

Microelectrodes

Single-barreled glass microelectrodes for extracellular recordings were pulled from thin-walled borosilicate glass (GC150TF-10, Harvard Apparatus) using a horizontal puller (P-97 Flaming/Brown Micropipette Puller, Sutter Instruments). They were filled with ACSF and their tips were broken to a final resistance of 5–10 MΩ. Sharp microelectrodes for current-clamp recordings were made from thick-walled borosilicate glass (GC150TF-10, Harvard Apparatus) and filled with 2 M K-acetate + 5 mM KCl +10 mM HEPES; pH 7.4. Their resistances were ~80 MΩ.

Hypoxia-protocol and electrical recordings

Severe hypoxia was induced by switching the recording chamber’s gas supply from carbogen (95% O₂-5% CO₂) to 95% N₂-5% CO₂, while the carbogen aeration of the experimental solutions was continued. Such treatment resulted within a few minutes in the occurrence of HSD. The time point of reoxygenation critically determines the reversibility of the hypoxia-induced changes. Therefore we resubmitted oxygen soon (15–20 s) after the onset of HSD, which was indicated by a sudden drop in extracellular DC potential. Within that time the extracellular DC potential shift had reached its nadir.

All signal amplitudes were measured between the prehypoxia baseline and the maximal change. Only rapid negative extracellular DC potential changes (ΔVₜₐₜ) of ≥10-mV amplitude were considered as HSD, and only those slices were accepted for the experiments, which produced an HSD no earlier than 90 s after oxygen withdrawal (no earlier than 60 s in Ca²⁺-free solutions). HSD onset was defined as the occurrence of the sudden ΔVₜₐₜ.

Evoked responses were elicited by 0.1-ms unipolar stimuli delivered via microwire electrodes made from bare stainless steel wire (50-µm diam, AM-Systems) and recorded as described in detail earlier (Müller and Somjen 1998). Orthodromic responses were elicited by stimulation of Schaffer collaterals and recorded in stratum radiatum of the CA1 region; antidromic responses were elicited by stimulating pyramidal cell axons in the alveus and recorded in s. pyramidal of the CA1 region. All extracellular recordings were performed with a locally constructed extracellular DC potential amplifier.

Current-clamp recordings from CA1 neurons were performed with an intracellular recording amplifier (SEC-05L, NPI Instruments, Tamm, Germany). Bridge balance and electrode-capacitance compensation were adjusted before insertion of the electrode and continuously controlled during the entire recording. CA1 pyramidal neurons were identified by their location in s. pyramidal, membrane potential, spontaneous activity, action-potential shape and input resistance (Morin et al. 1996). Glial cells (astrocytes) lining the pyramidal cell layer were identified by their negative membrane potential of −80 to −90 mV, low input resistance, the absence of spikes in response to depolarizing stimuli (D’Ambrosio et al. 1998). Successful cell impalement was achieved by slowly advancing the electrode into the slice and applying a brief high-frequency current pulse (“buzz”) to the electrode. Only CA1 neurons and glial cells with stable membrane potentials of at least −55 and −80 mV, respectively, were accepted. Neuronal input resistance was probed every 10 s by a hyperpolarizing current of 400- to 600-pA amplitude and 300-ms duration. Data were sampled at 2.5 kHz using a TL-1/Labmaster acquisition system and the Axotape V2 software (Axon Instruments). Input resistance was measured at the steady-state level of the voltage deflections and averaged over 10 successive current injections. Changes in input resistance were expressed as percent of pretreatment value.

Optical recordings

Optical recordings of intracellular Ca²⁺ concentrations, mitochondrial membrane potential, and NADH/FAD autofluorescence were performed with a standard computer-controlled fluorescence imaging system composed of a monochromatic light source (Polychrome II; Till Photonics, Gräfelfing, Germany) and a highly sensitive CCD camera (Imago QE, Pico Imaging, Kelheim, Germany) attached to an upright microscope (Axioptec vario, Zeiss, Oberkochen, Germany). The computer controlled monochromatic light source selects the predefined wavelength(s) by a galvanometer-mounted grating that projects the desired range of the spectrally dispersed light onto a
narrow slit. Therefore the final excitation light shows a Gaussian wavelength distribution around the selected center-wavelength; bandwidth at half-maximum is 15 nm. In the following, just the center wavelengths of excitation light are mentioned. Rh123 was excited at 485 nm and fluorescence was recorded using a 505-nm beamsplitter and a 535/35-nm band-pass filter. Cellular NADH and FAD were excited alternately at 360 nm (NADH) and 460 nm (FAD), which are close to their respective absorption maxima, and the resulting autofluorescence was recorded simultaneously using a 505-nm beamsplitter and a 510/40-nm band-pass filter. This beamsplitter/emitter constellation used absorbed a large part of NADH autofluorescence (which is highest around 400–450), yet it made possible the simultaneous detection of NADH and FAD autofluorescence. For imaging experiments, tissue slices and cell cultures were placed in a submersion-style chamber at 33–35°C.

Statistics

The data were obtained from ~70 rats, and because most experiments did not last longer than 1–2 h, up to five slices could be used from each brain. However, to ensure independence of observations, each experimental treatment was performed on at least three different rats. All numerical values are represented as means ± SD. Significance of the observed changes was tested using a two-tailed, unpaired Student’s t-test and a significance level of 5% (unpaired observations). The plotted parameters are normalized to the respective control parameters of a 2nd HSD induced in an untreated slice. For statistical comparison, the parameters of a 2nd HSD induced in an untreated slice are plotted as well. Error bars represent SDs; *, significant changes as compared with this 2nd HSD induced in an untreated slice, i.e., the data set 2nd hypoxia plotted on the very right (*P < 0.05; **P < 0.01; n, number of trials).

RESULTS

Effects on hypoxia-induced SD

Severe hypoxia induces within a few minutes a nearly complete depolarization of hippocampal neurons and glial cells, which is associated by the occurrence of a negative shift in the extracellular DC potential, termed hypoxia-induced spreading depression (for review, see: Somjen 2001, 2004). In untreated slices HSD occurred within 156.6 ± 54.5 s, the associated negative shift of the extracellular DC potential (ΔV_{EC}) averaged −16.1 ± 4.2 mV and measured at its half-amplitude level it lasted 45.6 ± 10.4 s (n = 115). As we have shown previously, HSD of short duration can be induced repeatedly in a given slice, and with a sufficient recovery time of ~30 min in between the hypoxic episodes, the characteristic parameters are not significantly affected for the first three HSD episodes (Müller and Somjen 1998).

Modulation of sulfhydryl groups did, however, result in characteristic changes of HSD. As compared with the previously induced control HSD, application of the sulfhydryl oxidizing agent DTNB (2 mM, 20 min) postponed the onset of HSD by 29.2 ± 18.8% and decreased its amplitude by 15.7 ± 8.8% (n = 9); HSD duration was not affected (Fig. 1, A and C). By contrast, treatment with the SH-reducing agent DTT (2 mM, 20 min) mimicked the effects of DTNB, also postponing HSD. The amplitude of the plotted extracellular voltage deflection (ΔV_{EC}) decreased slightly, whereas its duration (measured at the 50% level) was unaffected. Severe hypoxia was induced by switching the recordings chambers gas supply from carbogen to 95% N₂–5% CO₂. B: the sulfhydryl reducing agent 1,4-dithio-DL-threitol (DTT, 2 mM, 20 min) shortened the time to HSD onset but did not affect its duration. C: statistical summary of sulfhydryl-modulator induced changes. Similar to DTT, N-ethylmaleimide (NEM) accelerated the onset of HSD and in addition it decreased its duration, whereas application of H₂O₂ (1 mM, 20 min) mimicked the effects of DTNB, also postponing HSD. The plotted parameters are normalized to the respective control HSD previously induced in each slice. For statistical comparison, the parameters of a 2nd HSD induced in an untreated slice are plotted as well. Error bars represent SDs; *, significant changes as compared with this 2nd HSD induced in an untreated slice, i.e., the data set 2nd hypoxia plotted on the very right (*P < 0.05; **P < 0.01; n, number of trials).
mM, 20 min) shortened the time to HSD onset by 25.0 ± 17.3% and decreased its amplitude by 26.1 ± 14.9% (n = 8). The duration of HSD was not affected (Fig. 1, B and C). The sulfhydryl alkylating agent NEM (500 μM, 20 min) shortened the time to HSD onset by 48.4 ± 10.4% and reduced its duration by 29.9 ± 5.5%; HSD amplitude was not affected (n = 7, Fig. 1C). Application of the rather unspecific oxidizing agent H₂O₂ (1 mM, 20 min) caused a moderate, less consistent postponement of HSD by 12 ± 19% (n = 7).

In general, treatments increasing neuronal excitability facilitate the generation of SD, whereas those decreasing excitability postpone its occurrence. So it seems that DTT might activate the generation of SD, whereas those decreasing excitability K⁺ channels, glutamate receptors, and K⁺ channels in the DTNB- and DTT-induced modulation of HSD. In these experiments, the control HSD was already induced in the presence the given blocker and the second HSD was elicited in the continued presence of the blocker plus DTNB or DTT.

Withdrawal of extracellular Ca²⁺ (n = 7, Fig. 2, A and C) or application of 2 mM Ni²⁺ (n = 5, Fig. 2, B and C) prevented the DTNB-induced postponement of HSD, indicating the requirement of Ca²⁺ influx from extracellular space for the delayed HSD onset. In the presence of the NMDA antagonist dL-AP5 (150 μM, n = 6) or the AMPA/kainate antagonist CNQX (25 μM, n = 8), the DTNB-induced postponement of HSD remained unchanged (Fig. 2C).

The postponement of HSD by DTNB could indicate an increased activity of K⁺ channels. We therefore tested for the DTNB-sensitivity of different K⁺ channel types. Low concentrations of TEA (500 μM, n = 6), which are assumed to target BK channels (Beck et al. 1997), or treatment of slices with the K⁺ATP channel blocker tolbutamide (200 μM, n = 6) did not affect the DTNB-induced postponement of HSD (Fig. 3B). However, pretreating slices with 20–25 nM charybdotoxin (CTX, n = 5 Fig. 3A) or 50 nM iberiotoxin (IBX, tested in 3 trials only) consistently abolished the DTNB-induced postponement of HSD (Fig. 3, A and B). It therefore seems that sulfhydryl modulation activates Ca²⁺-activated, high-conductance K⁺ channels of the BK type.

Analogous pharmacological experiments were also performed to identify the target of DTT. Combination of Ca²⁺-free solutions with DTT (2 mM) triggered within 19.7 ± 4.7 min (n = 4) spontaneous recurrent SD episodes that irreversibly damaged the slices under investigation. Subsequent oxygen withdrawal failed to trigger full-size HSDs in these slices (Fig. 4A). One slice that did not produce spontaneous SDs under these conditions showed a further reduction in HSD onset by 50% in DTT. Ni²⁺ could not be tested because it formed a massive brown precipitate with DTT. In the presence of the glutamate antagonists CNQX (25 μM, n = 5) or dL-AP5 (150 μM, n = 5), the DTT-induced hastening of HSD onset was unchanged and tolbutamide (200 μM, n = 5) did not prevent this effect of DTT (Fig. 4B). Because the peptidergic BK channel blockers charybdotoxin and iberiotoxin each possesses three disulfide bonds, which are potential targets for reduction by DTT, we rather chose the nonpeptidergic BK channel inhibitor paxilline (Sanchez and McManus 1996). In our trials, paxilline (10 μM) did not prevent the hastening of HSD onset by DTT (n = 5, Fig. 4B), and it also failed to...
prevent the postponement of HSD by DTNB (n = 4, Fig. 4B). It therefore seems questionable whether paxilline is effective under our experimental conditions at all.

Changes in cellular excitability and synaptic function

To explore whether DTNB and DTT affect other neuronal functions that are likely to be influenced by BK channels, we performed sharp electrode recordings from single CA1 neurons and glial cells in interfaced slices. In addition, we probed for changes in synaptic function by recording extracellular field potentials.

Because pyramidal cells and interneurons respond to hypoxia identically (Müller and Somjen 2000a), the data of both types of cells were pooled (11 pyramidal cells, 9 interneurons). Average membrane potential and input resistance of the recorded neurons were $-65.9 \pm 5.0$ mV and $25.5 \pm 7.5$ MΩ (n = 20). In untreated control slices, oxygen withdrawal triggered within 1–2 min an initial hyperpolarization of CA1 neurons that then turned into a slow depolarization and finally triggered (after $177 \pm 69$ s, n = 7) an explosive depolarization close to 0 mV (for details, see Müller and Somjen 2000a,b) (Fig. 5A).

Application of DTNB (2 mM, 20–25 min) caused a moderate hyperpolarization, by $3.8 \pm 2.8$ mV and decreased the input resistance, by $17.9 \pm 13.2\%$ (n = 6). As a result, spontaneous spike discharges were reduced or even ceased (Fig. 5B). In the presence of DTNB, oxygen withdrawal triggered the anoxic depolarization within $237 \pm 64$ s (n = 7), i.e., the CA1 neurons tolerated hypoxia longer than untreated control cells. The initial hyperpolarization was slightly reduced or was even absent, probably due to the more negative resting membrane potential in the presence of DTNB (Fig. 5B).

Incubation with DTT (2 mM, 20–25 min) caused a moderate depolarization in four cells and a moderate hyperpolarization in the two others; the input resistance did not change significantly (Fig. 5C). However, in the presence of DTT spontaneous spike discharges became more frequent, and within $<15$ min well-pronounced burst discharges developed (Fig. 5D). During these burst discharges single spikes became progressively broadened (Fig. 5D). In the presence of DTT, oxygen withdrawal triggered the massive hypoxic depolarization within $97 \pm 45$ s. The initial hypoxic hyperpolarization was usually blocked, and the neurons directly started to depolarize slowly when oxygen was withdrawn (Fig. 5C).

By incidence in hippocampal cell cultures, we observed the retraction of glial processes in response to DTT (Fig. 6A), whereas neuronal processes remained intact. This retraction started within 7–10 min of DTT application and was completed within another 30–40 min. DTTN application did not cause such changes in cell shape. To elucidate, whether DTT disturbs glial function, which would severely interfere with neuronal and synaptic function, we performed sharp electrode recordings from CA1 glial cells in hippocampal slices. The average glial membrane potential was $-89.7 \pm 5.6$ mV (n = 6); their input resistance was too low to be probed reliably. In response to DTT glial cells showed a slow but continuous depolarization which averaged $19.2 \pm 7.5$ mV after 15–20 min of DTT treatment (Fig. 6B, n = 6). The synchronized burst discharges elicited by DTT in pyramidal neurons, caused—apparently due to an increased extracellular K+ concentration—slow transient depolarizations of glial cells by $\pm 20$ mV (Fig. 6B). Continuing DTT treatment beyond 30 min usually caused spontaneous SD episodes during which glial cells underwent the characteristic massive depolarization (see also Müller and Somjen 2000a).

Orthodromically evoked field potentials (fEPSPs) and antidromically evoked population spikes were recorded in s. radiatum and s. pyramidale of the CA1 region, respectively. Compared with the evoked responses previously recorded in the same slice, DTNB (2 mM, 20 min) tended to somewhat reduce the amplitude of evoked excitatory field potentials (Fig. 7A). Paired-pulse facilitation was not significantly affected (Fig. 7A). Against expectation, DTT (2 mM, 20 min) depressed orthodromically evoked fEPSPs by 45–50% (n = 6), but it also induced multiple population spikes in response to single orthodromic stimuli (Fig. 7B). Paired-pulse facilitation was also somewhat reduced by DTT.
This depression of fEPSPs by DTT also occurred in the continued presence of 3 mM glutamine (Fig. 7C, n = 6). Axonal conduction was not affected by either DTNB or DTT as judged by the unchanged amplitudes of antidromic population spikes (Fig. 8). Yet again, DTT induced multiple population spikes in response to a single stimulus (Fig. 8B).

Is mitochondrial function modulated?

Because direct effects of DTNB and DTT on mitochondria would be expected to modulate neuronal susceptibility to hypoxia, we elucidated whether these compounds affect mitochondrial membrane potential and/or metabolism. In detail, we probed for changes in rhodamine 123 (Rh123) fluorescence, a marker for mitochondrial membrane potential (ΔΨ), and for changes in autofluorescence, representing the cellular redox couples NADH/NAD⁺ and FADH₂/FAD. These recordings were performed in a submersion style chamber (33–35°C).

In Rh123 labeled slices (2–5 μg/ml Rh123 loading for 25 min) as well as in cultured CA1 neurons, the protonophore FCCP (1–2 μM) induced a clear increase in Rh123 fluorescence, 65 ± 21% in s. radiatum of acute slices (n = 7) and 63 ± 33% in the cytosol (soma) of cultured CA1 neurons (n = 8), thereby indicating pronounced mitochondrial depolarization (Fig. 9, A and B). In contrast, DTNB and DTT (2 mM each, applied for 7–9 min) did not markedly affect mitochondrial...
drial membrane potential—neither in cultured CA1 neurons nor in acute hippocampal slices (Fig. 9, A and B). Also, recording autofluorescence from acute slices did not reveal any pronounced effects of DTNB or DTT (2 mM each) on the cellular levels of NADH and FAD. Autofluorescence excited at 360 nm represents reduced NADH, whereas autofluorescence excited at 460 nm corresponds to oxidized flavins (FAD). As expected, in response to mitochondrial inhibition by anoxia, 1 mM CN⁻/H₁₁₀₀₂ caused opposite changes in these simultaneously recorded measures (Duchen and Biscoe 1992), shifting NADH to its reduced and FAD to its oxidized form (Fig. 9, C and D). Accordingly, NADH autofluorescence increased by 9.2 ± 1.9%, while FAD autofluorescence decreased by 7.4 ± 1.8% (n = 6 each). DTT induced only barely noticeable changes in autofluorescence levels. DTNB, which absorbs light in the range of 300–450 nm, caused an artificial drop in NADH fluorescence due to competitive absorption of excitation light (Fig. 9D). The time course of these changes as well as the absence of a corresponding, opposite signal in FAD autofluorescence do confirm the artifactual nature of the DTNB-induced changes in NADH autofluorescence.

**DISCUSSION**

We demonstrated that sulfhydyl modulation affects the susceptibility of hippocampal tissue slices to the generation of the highly

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**FIG. 5.** Current-clamp recordings showing the effects of DTT and DTNB on single CA1 neurons during normoxia and subsequent hypoxia. A: the hypoxic response of untreated control cells consists of an initial hyperpolarization that is followed by the regenerative, nearly total depolarization. On reoxygenation the cells start to recover, but during that phase, intracellular recordings usually become unstable. Numbering refers to the characteristic points of the hypoxic response. 1, resting membrane potential; 2, initial hyperpolarization; 3, ignition threshold; 4, peak of fast depolarization; 5, absolute peak. Their values are summarized in E for the various treatments. B: DTNB treatment (2 mM, 20 min) caused a moderate hyperpolarization and abolished spontaneous spike discharges, in parallel the input resistance decreased slightly. The anoxic depolarization was postponed by DTNB, but otherwise occurred unchanged, in an all-or-none fashion. C: DTT application (2 mM, 20 min) resulted in a moderate depolarization and an increasing rate of spontaneous spike discharges. The time to onset of the anoxic depolarization was shorter than under control conditions, and the initial hyperpolarization was less pronounced or even absent. D: in addition to more frequent spike discharges, DTT also induced pronounced burst firing. The displayed trace segments were recorded from the same neuron before and after 20 min DTT treatment. The trace displayed on the very right is a stretched segment of a burst discharge recorded in the presence of DTT (see arrow mark). E: summary of the characteristic potentials of the anoxic depolarization. For definition of potentials see numbering 1–5 in A. Marked differences did not occur in the presence of DTNB or DTT. Once the anoxic depolarization was triggered, it occurred in an all-or-none fashion. Compared with untreated control cells (n = 7), its onset tended, however, to be postponed by DTNB (n = 7) and hastened by DTT (n = 5).
synchronized anoxic response, i.e., HSD. Oxidation of sulfhydryl groups by DTNB or H$_2$O$_2$ postponed the onset of HSD, whereas their reduction by DTT as well as their alkylation by NEM hastened HSD onset. The Ca$^{2+}$/H$^{11001}$ dependence of the DTNB-induced postponement as well as its sensitivity to charybdotoxin and iberiotoxin prove that an enhanced activity of BK-type channels underlies the observed modulation of HSD. The mechanisms involved in the hastening of HSD onset by DTT are less clear.

**How is SD postponement achieved?**

The generation of HSD reflects the concerted activation of various types of ion channels and glutamate receptors enabling massive, self-regenerative Ca$^{2+}$ and Na$^{+}$ influx into neurons that is paralleled by K$^{+}$ release into extracellular space (Aitken et al. 1991; Müller and Somjen 1998, 2000b; Somjen 2001, 2004). Computer simulations, based on experimental data...
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FIG. 7. Sulfhydryl reduction markedly depresses synaptic efficacy. A and B: the plotted input-output curves as well as the sample recordings of excitatory postsynaptic potentials (EPSPs) show that DTNB tended to somewhat reduce EPSP amplitudes (n = 7), whereas DTT significantly depressed the amplitudes of orthodromically evoked responses (n = 6), indicating block of synaptic function on reduction of SH groups. Paired-pulse ratios of orthodromic responses, tested by twin pulses separated by 75 ms, were not affected significantly by either treatment. EPSPs were recorded in stratum radiatum of the CA1 region. The stimuli (0.1 ms, unipolar) are marked by the arrows; stimulation artifacts are truncated. C: the depression of synaptic function by DTT could not be prevented by glutamine (3 mM). Plotted EPSPs amplitudes are normalized to the pretreatment control amplitudes (100 μA, 0.1 ms stimulus), and they are compared for the effects of DTT in control and glutamine-treated slices (n = 6 each). Sample traces show EPSPs elicited in glutamine-containing ACSF before and after addition of DTT (17 min, 2 mM).

FIG. 8. Antidromic population spikes, i.e., axonal conduction, is not depressed by sulfhydryl modulation. A: significant changes in the antidromically evoked population spikes were not observed during DTNB treatment (n = 6). B: while the amplitude of antidromic responses was unaffected (n = 5), stimulation in the presence of DTT evoked multiple population spikes, indicating enhanced excitability of CA1 pyramidal neurons on reduction of SH groups.
Kager et al. (2002), as well as current source density analyses (Wadman et al. 1992) provided evidence that SD is ignited in the dendrites. The ignition point is reached, when the sum of all inward currents exceeds the total outward current, i.e., when the net dendritic current turns inward (Kager et al. 2002).

Inhibition of voltage-gated Na\(^+\)/H\(^+\) channels, Ca\(^{2+}\)/H\(^+\) channels, or glutamate receptors does not prevent SD but just postpones its onset. No matter what maneuvers are taken, in single neurons the anoxic depolarization, once triggered, evolves until completion in an all-or-none fashion (Müller and Somjen 2000b; Kager et al. 2002). If the hypoxic depolarization is less synchronized within the neuronal population, this might result in a reduced extracellular DC potential shift but again without dampening the massive depolarization in individual pyramidal cells and interneurons. But how does oxidation of SH groups and the resulting postponement of HSD onset fit into this scenario?

The immediate, early response of hippocampal CA1 neurons to hypoxia is an initial hyperpolarization, which is generated by activation of K\(^+\) channels and subsequent K\(^+\) release into interstitial space (Hansen et al. 1982; Donnelly et al. 1992; Müller and Somjen 2000a). Whether solely BK-type channels or K\(_{ATP}\) channels are activated or whether both types of channels are involved, with their contribution being weighed depending on the severity of an insult and the experimental conditions, is still under discussion (Erdemli et al. 1998; Fujimura et al. 1997; Zawar and Neumcke 2000). Under our experimental conditions (slices of adult rats placed in an interface chamber at 35–36°C), the initial hyperpolarization seems to be mediated mostly by BK-type channels: DTNB was found to hyperpolarize CA1 neurons by activation of BK channels and subsequent oxygen withdrawal did not cause a further hyperpolarization of these cells. That the postponement of HSD by DTNB was abolished by charybdotoxin, iberiotoxin, Ni\(^{2+}\)/H\(^+\), and Ca\(^{2+}\)/H\(^+\) withdrawal further supports the activation of BK channels. By contrast, tolbutamide was without effect, ruling out an involvement of K\(_{ATP}\) channels—at least during the early phase of hypoxia. Also the time to onset of HSD in untreated control slices (167 ± 63 s, n = 19) and in slices
treated with 200 μM tolbutamide (143 ± 42 s, n = 12) did not differ.

We therefore conclude that the DTNB-mediated oxidation of SH groups activates BK channels, thereby postponing the onset of HSD. Whether DTNB directly acts on BK channels—modulating their gating properties or rendering them more Ca²⁺ sensitive—cannot be decided yet on the basis of our data. Indications for an increased Ca²⁺ sensitivity of hippocampal BK channels were found during the posts ischemic period (Gong et al. 2002). Alternatively, DTNB could as well modulate voltage-gated Ca²⁺ channels and enhance Ca²⁺ influx during the initial phase of hypoxia, thereby activating BK channels indirectly. Such an activation of Ca²⁺ channels, leading to subsequent activation of a charybotoxin-sensitive KCₐ current, was reported in dissociated hippocampal neurons by Nowicky and Duchen (1998) in response to cyanide poisoning of mitochondria or their uncoupling by FCCP. The fact is that in our experiments Ca²⁺ influx from extracellular space was required for the activation of BK channels and that it was carried via voltage-gated Ca²⁺ channels, not via glutamate receptors, because DL-AP5 and CNQX failed to antagonize the DTNB-induced postponement of HSD.

The different time course of BK channel activation in the presence of DTNB or the enhanced BK-mediated outward current resulted in a postponement of SD. This could be due to more efficient glial K⁺ buffering, which was demonstrated in computer simulations to postpone or even prevent SD (Somjen 2004; p. 309). BK channels were already open before the onset of hypoxia, thereby causing an earlier hyperpolarization and allowing the neurons to withstand the generation of SD for a longer time period. Interestingly, BK channels were also reported to control the generation of Ca²⁺ spikes in pyramidal cell dendrites (Golding et al. 1999). Accordingly, an increased BK channel activity could successfully shift the ratio of inward versus outward current more to favor of the outward current, thereby preventing dendritic depolarization and thus HSD ignition within the dendritic tree just a little bit longer. As BK channels are assumed to be expressed in astrocytes as well (Barres et al. 1990), they could facilitate K⁺ uptake and K⁺ buffering within the glial synctium. However, because ignition of SD occurs in neurons while glial cells just follow passively the extracellular increase in K⁺ (Müller and Somjen 2000a; Somjen 2001), we rather believe that the postponement of HSD due to modulation of BK channels is more likely to take place in neurons rather than glia.

Redox modulation of hippocampal circuitry and excitability

DTNB tended to decrease synaptic efficacy only slightly, while axonal conduction was not affected (Figs. 7A and 8A). The slight dampening of synaptic function is probably the result of the moderate hyperpolarization of single CA1 neurons in response to DTNB, i.e., a reduced postsynaptic excitability due to the increased resting K⁺ conductance or a damping effect of DTNB on the NMDA component of the EPSP (Tauck 1992). Paired-pulse facilitation, which arises from residual Ca²⁺ remaining in the presynaptic terminal after the first stimulus (Zucker 1989), was not reduced by DTNB, suggesting that presynaptic Ca²⁺ cycling was not affected.

By contrast, DTT markedly reduced synaptic efficacy but did not depress antidiromic responses. In addition, it increased postsynaptic excitability (Figs. 7B and 8B). The moderate depolarization observed in single hippocampal neurons and the induction of burst discharges by DTT (Fig. 5D) correspond to earlier reports obtained in guinea pigs (Tolliver and Pellmar 1988), and they indicate an enhanced excitability of CA1 neurons. Obviously these changes in CA1 neurons are also responsible for the firing of multiple population spikes in response to single antidiromic stimuli (Fig. 8B) as well as for the observed depolarization of glial cells (Fig. 6B), which suggests an increase in extracellular K⁺. Thus the observed depression of synaptic function rather seems to be due to presynaptic effects of DTT in the Schaffer collateral terminal. If also induced presynaptically, the DTT-mediated depolarization or the increased extracellular K⁺ could inactivate presynaptic Na⁺ and Ca²⁺ channels, thereby reducing Ca²⁺ influx and transmitter release from the synaptic terminals (Eccles et al. 1963; MacDermott et al. 1999). As glutamine failed to prevent synaptic depression by DTT, glial poisoning by DTT can be excluded as a possible explanation for synaptic failure.

Besides the proven modulation of BK channels, additional channels and receptors are known to be sensitive to sulphhydryl modulation. DTT increases, while DTNB decreases NMDA currents (Sanchez et al. 2000; Tauck 1992), thereby enhancing NMDA receptor-mediated toxicity in hippocampal slice cultures (Pringle et al. 2000). In contrast, DTNB suppresses spontaneous ictal activity due to inhibition of NMDA receptors (Sanchez et al. 2000). Yet for our data, redox modulation of glutamate receptors can be ruled out because the effects of DTNB and DTT on HSD persisted in the presence of NMDA and non-NMDA antagonists (Figs. 2C and 4B).

Persistent Na⁺ channels are potentiated by hypoxia (Hammarström and Gage 1998) and an increase in extracellular K⁺ (Somjen and Müller 2000), and they play a key role in the triggering of normoxic SD and HSD (Kager et al. 2002; Somjen 2001). Interestingly INa,p is also sensitive to SH modulation, being increased by NO donors in a DTT-sensitive manner (Hammarström and Gage 1999). Nevertheless, persistent Na⁺ channel modulation cannot explain our findings either because it was BK-channel inhibition that abolished the DTNB-mediated postponement of SD. Also, if Na⁺ channels were modulated by DTNB or DTT, one would expect effects on axonal conduction as well. Antidiromic responses were, however, found not to be modulated by either DTT or DTNB (Fig. 8).

Changes in cellular redox state during hypoxia

The oxidation of SH groups was found to postpone HSD. Severe hypoxia itself causes a decrease of mitochondrial metabolism by inhibiting complex IV of the respiratory chain, which should decrease the formation of ROS (Boveris and Chance 1973; Votyakova and Reynolds 2001). In addition, the redox couple NAD⁺/NADH is shifted even more to the reducing form, thus shifting the cellular redox status toward reducing conditions (Mills and Jöbsis 1972; Riepe et al. 1996) (Fig. 9). In contrast, reoxygenation, rotenone, antimycin, Ca²⁺-induced mitochondrial depolarization, increased ATP-synthesis and mitochondrial uncoupling by, e.g., FCCP increase ROS production (Bindokas et al. 1996; Boveris and Chance 1973).

Could reducing conditions possibly favor the generation of HSD? NMDA receptor-mediated currents are increased under
findings, Nowicky and Duchen (1998) reported activation of homeostasis and especially the massive Ca\(^{2+}\) signaling the membrane potential and preventing the ignition of activity already before oxygen withdrawal succeeded in stabilizing mitochondria. Specifically impaired mitochondria finally affects membrane conductances. The fact is that neurons respond immediately to metabolic compromise, and they respond—with protective mechanisms—way before cellular ATP is depleted (Hansen et al. 1982; Lipton and Whittingham 1982; Müller et al. 2002). The detailed molecular events involved in the sensing of hypoxia by central neurons are only partly understood, but it seems that SH modulation may play a pivotal role in these processes.

Neuroprotective potential of SH oxidation

The postponement of HSD was found to depend on the activation of BK channels. In general, these channels decrease neuronal excitability, and others reported them to reduce cell death from ischemic insults in CA1 pyramidal cell cultures and to regulate presynaptic glutamate release (Runden-Pran et al. 2002). We found that—even though BK channels are activated already during the early phase of hypoxia—their increased activity before oxygen withdrawal succeeded in stabilizing the membrane potential and preventing the ignition of HSD for some more time—on average 30\% longer than in untreated slices. As a result, the dramatic disturbance of ionic homeostasis and especially the massive Ca\(^{2+}\) load being associated with the hypoxic depolarization (Hansen 1985; Martin et al. 1994; Müller and Ballanyi 2003) were delayed. The decreased amplitude of HSD might indicate desynchronization of the anoxic depolarization in CA1 neurons. Both postponement and desynchronization may prevent HSD during short-term insults and possibly also reduce its spreading speed and range. Accordingly, parts of the neuronal population could be affected later or might even be spared from the loss of membrane potential and the threatening Ca\(^{2+}\) overload. Stroke is of course rarely anticipated, but peri-infarct SD waves reaching out in the penumbra and aggravating neuronal damage of surrounding tissue that was initially not affected (Busch et al. 1996; Kemptsi et al. 2000) could be dampened or depressed by well-directed SH modulation. Therefore oxidizing SH groups might mediate neuroprotection during metabolic insults.

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